



The Assessment of HE4 in Premalignant and Malign Urothelial Tumors

Betul Celik^{1*} and Tangul Bulut¹

¹Department of Pathology, Antalya Hospital, Health Sciences University, Defterdarlik Blv,
Antalya, 07050, Turkey.

Authors' contributions

This work was carried out in collaboration between both authors. Author BC designed the study, wrote the protocol, and the first draft of the manuscript. Author TB performed the statistical analysis, helped the analyses of the results. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMMR/2018/45373

Editor(s):

(1) Dr. Evangelos Marinos, Associate Professor, University of Athens, School of Medicine, Laboratory of Biology, Athens, Greece.

Reviewers:

(1) Antonio Augusto Ornellas, Brazilian National Institute of Cancer, Brazil.
(2) Mahesh Deshmukh, Rashtra Sant Tukadoji Regional Cancer Center, India.
(3) Hans Olsson, Linkoping University Hospital, Sweden.
Complete Peer review History: <http://www.sdiarticle3.com/review-history/45373>

Received 26 September 2018

Accepted 02 January 2019

Published 18 January 2019

Original Research Article

ABSTRACT

Background: The aim of this study was to evaluate the expression and the prognostic significance of the Human Epididymis Protein 4 (HE4) in urothelial tumors of the bladder.

Materials and Methods: The current study included 55 patients with a histopathological diagnosis of urothelial neoplasm obtained from transurethral resection between 2010 and 2016. The expression of HE4 was examined using immunohistochemical methods.

Results: There was 5 papillary urothelial neoplasia of low malignant potential (PUNLMP); 16 low grade non-invasive papillary urothelial carcinoma (LGUC); 7 high grade non-invasive papillary urothelial carcinoma (HGUC); 18 lamina propria-invasive urothelial carcinoma (invUC); and 9 muscle-invasive urothelial carcinomas (musc-invUC). Of the total, 20% LMP, 6.2% LGUC, 14.2% HGUC, 28.5% invUC and 33.3% musc-invUC cases were successfully stained with HE4 immunohistochemically. The overall expression of HE4 among urothelial tumors was 18.2%.

Conclusions: The Human Epididymis Protein 4 expression is proportionally higher in invasive urothelial neoplasm but the difference is not statistically significant between invasive vs non-invasive tumors. We propose that it can be used for the assessment of invasion status when the bladder muscle is not sampled.

*Corresponding author: E-mail: betul.celik@sbu.edu.tr, bet_celik@yahoo.com;

Keywords: HE4; bladder; transitional; carcinoma; papillary.

1. BACKGROUND

Bladder cancer (BC) has been the most prevalent urinary tract malignancy in the USA. The incidence is of 79,030 cases and 16,870 deaths in 2017 [1]. The predominant histologic type is urothelial (transitional) cell carcinoma, which includes papillary lesions, carcinoma in situ (CIS), and invasive tumors. Two potential pathways have been reported for BC development: Low-grade papillary tumors that contain oncogenic mutations in FGFR or HRAS, and high-grade/invasive tumors that have defects in the tumor suppressor pathways such as p53 and retinoblastoma (RB) [2,3]. Papilloma, papillary urothelial neoplasm of low malignant potential (PUNLMP), and LGPUC are low-grade papillary tumors that recur frequently but rarely progress, whereas high-grade invasive tumors are usually diagnosed at advanced stage. Recent studies revealed the more complex molecular subclasses that may provide new opportunities for prognostic application and personalized therapy [4].

Human epididymis 4 (HE4) protein belongs to whey acidic 4-disulfide center protein family [5]. It is a protease inhibitor and is involved in the innate immune defense of the respiratory tract and nasal cavity [6]. It was first described in epididymis but subsequent studies revealed that it was present in different tissues and cancers [7]. Its higher level in the serum predicts poor prognosis in lung adenocarcinoma and epithelial ovarian cancer patients [8,9]. Previous studies of our study group showed its presence in the ovarian, lung, and gastric carcinoma cells as well [10,11,12].

In this study, we aimed to examine the presence of HE4 expression in human urothelial tumors and if present, its sequential potential toward PUNLMP, LGPUC, HGPUC, and invasion steps.

2. MATERIALS AND METHODS

After obtaining approval from institutional Ethics Committee, a total of 163 patients who had a diagnosis of PUNLMP, LGPUC, and HGPUC after initial transurethral resection (TUR) for bladder neoplasm between 2010 and 2016 at our institution were retrospectively enrolled in this study. The histologic classification of tumors was made on the basis of guidelines from The 2016 WHO Classification of Tumours of the Urinary System [13]. On the other hand, cases

from urothelial proliferation described in 2004 WHO classification, which is now classified as "urothelial proliferation of uncertain malignant potential" were not included in the study. For each case, one representative tumor block containing sufficient tumor tissue was chosen. While taking section for immunohistochemistry, an extra section was taken and stained with H&E. Exclusion criteria were tumors with <10 tumor cells and tumors from metastatic foci. Cases in which clear-cut evidence of invasion was not seen were also excluded from the study. Patient information and histopathological parameters of each patient were obtained from the relevant pathology reports and from the hospital data basis. Tissue sections of normal human epididymis processed in a comparable manner provided as a positive control. Negative controls were obtained by omitting the primary.

2.1 Immunohistochemical Procedure

Formalin-fixed, paraffin-embedded sections were de-waxed with xylene and rehydrated through gradient ethanol into a phosphate buffered solution (PBS). Endogenous peroxidase activity was quenched with 0.3% H₂O₂ in methanol for ten minutes at room temperature. At the same time, 2 ml Tris-EDTA Buffer (abcam, ab93684) was added to 198 ml of distilled water, and swirled. Prepared retrieval solution was added to the microwaveable vessel. When the time elapsed, slides were washed in PBS three times and placed *into* the microwaveable vessel. The vessel was placed inside the domestic microwave, set to full power for 10 minutes, at a second highest power for 5 minutes and at medium power for 5 minutes. The procedure was monitored *for evaporation and watched for boiling over during the procedure and did not allow the slides to dry out*. When the retrieval solution evaporated *during the boil, hot* retrieval solution was added. When 20 minutes elapsed, the vessel was removed. When it cooled, the slides were washed in PBS 3 times before application of the rabbit polyclonal antibody to HE4 (Anti-HE4 antibody [EPR16658] [ab200828], 1:2000 dilution). After two hours incubation with the primary antibody, the slides were washed in PBS and biotinylated goat anti-rabbit IgG secondary antibody was applied and incubated for 10 minutes at room temperature. Slides were washed 3 times in PBS and Streptavidin Peroxidase was applied for 10 minutes at room temperature. At the same, time 20µl DAB

Chromogen was added to 1 ml of DAB Substrate and swirled. When the time elapsed, the slides were washed in PBS 3 times and prepared chromogen was applied to the tissues for 10 minutes at room temperature. Slides were then washed in PBS 3 times and lightly counterstained with hematoxylin, followed by dehydration and coverslip mounting. The tissue sections of the human epididymis were processed in a comparable manner and provided a positive control. The negative control was obtained by omitting the primary antibody (Fig. 1G). Cytoplasmic staining was graded for intensity (0-negative, 1-weak, 2-moderate, and 3-strong) and percentage of positive cells (0, 1 (1–24%), 2 (25–49%), and 3 (50–100%). The grades were multiplied to determine an H-score. Protein expression was then defined as negative (H-score=0), weak (H-score=1–3), or strong (H-score \geq 4).

2.2 Statistical Analysis

Data were evaluated by using SPSS ver. 11.5 (Chicago, INC.) programme. Chi-Square test and Fisher-Exact test were used to compare groups for categorical data. Oneway ANOVA and T test for independent samples were used to compare groups for age.

As descriptive statistics, mean \pm standart deviation was given to explain for continuous data, frequencies and percentages were given for categorical data. Statistical boundary was accepted 0.05.

3. RESULTS

One hundred sixty-three patients were retrieved from pathology archive between 2010 and 2016. Forty-five cases were excluded from the study, as pathology report did not mention the grade of the papillary tumor. While re-evaluating H&E slides, we noticed that representative part that allows us to classify a lesion was disappeared. This was especially common in LGPUC cases as well as PUNLMP and invasive part of the HGUCs. Overall, 63 cases were found ineligible for immunohistochemical staining. For the rest of 55 cases, 5 PUNLMP, 16 LGPUC, 7 HGPUC, and 27 invasive UC in which nine of them had muscularis propria invasion were found eligible and they were successfully stained with anti-HE4 antibody. There were 45 male (81.8%) and 10 female (18.2%) patients. Patients age was ranged between 40 to 89 years (mean 68.06 \pm 10.82).

Among 55 cases, the immunohistochemical assay indicated that one out of 5 PUNLMP (20%); one out of 16 LGPUC (6.2%); one out of seven HGPUC (14.2%); four out of 18 invasive UC (28.5%) and three out of nine (33.3%) muscle-invasive UC cases were successfully stained with HE4. Overall seven out of 27 (25.9%) invasive tumors were HE4 positive compared to three out of 28 (10.7%) non-invasive tumors. The staining intensity was weak (1+) in all except one HGPUC case (Fig. 1). The frequency of HE4 immunostaining between urothelial tumors was not significant statistically ($p=0.525$) (Fig. 2). When we adjusted tumors into invasive and non-invasive tumors, a difference was observed but it was statistically insignificant ($p=0.133$) (Fig. 3).

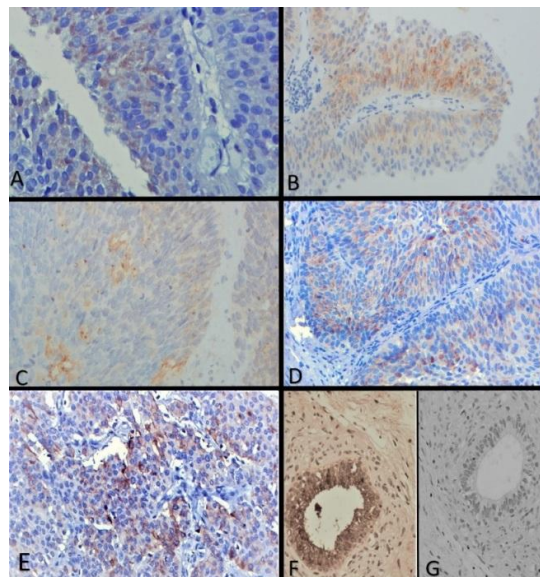


Fig. 1. Weak (1+) expression of HE4 in the cytoplasm of urothelial tumors
 A) PUNLMP, x400; B) LGPUC, x200; C) HGPUC, x200; D) Lamina propria invasive UC, x200; and E) muscularis propria invasive UC, x200; (anti-HE4. Positive and Negative controls are depicted in F and G respectively x200)

4. DISCUSSION

Bladder tumors are classified into two groups with distinct behavior and molecular profiles: Non-invasive tumors (generally papillary and usually superficial), and invasive (infiltrating) tumors [13]. Non-invasive tumors can progress with time to invasive carcinoma and the single most important factor for determining disease prognosis in bladder cancer is muscle invasion. Currently, there is no

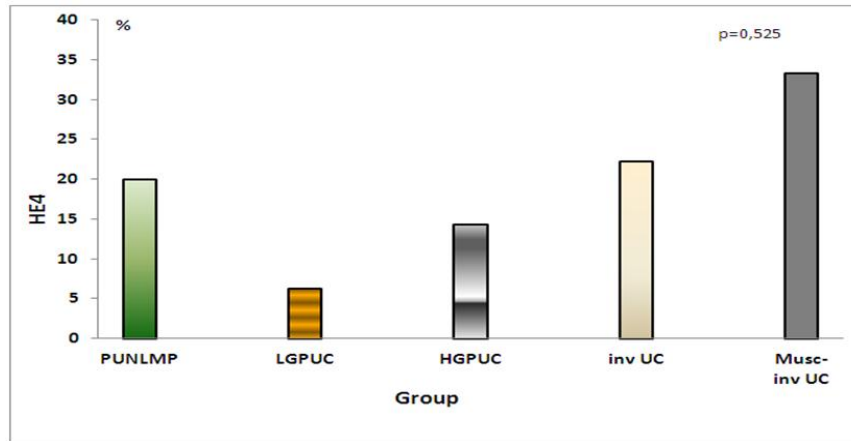


Fig. 2. Distribution of HE4 positivity among urothelial tumors groups

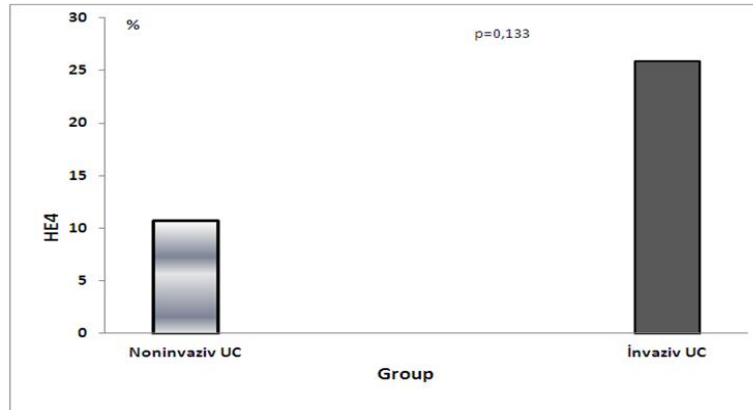


Fig. 3. Distribution of HE4 positivity between invasive and noninvasive urothelial tumors

Table 1. HE4 expression between groups

	Group 1 PUNLMP	Group 2 LGPUC	Group 3 HGPUC	Group 4 invUC	Group 5 Musc-invUC	P
N	5	16	7	18	9	
HE4 positive	1(%20,0)	1 (%6,2)	1 (%14,3)	4 (%22,2)	3 (%33,3)	0,525
HE4 negative	4 (%80,0)	15 (%93,8)	6 (%85,7)	14 (%77,8)	6 (%66,7)	

Table 2. HE4 expression between invasive versus non-invasive groups

	Noninvaziv UC	Invaziv UC	P
N	28	27	
HE4 positive	3(%10,7)	7(%25,9)	0,133
HE4 negative	25(%89,3)	20(%74,1)	

immunohistochemical marker available for the assessment of muscle invasiveness for bladder-TUR specimens in pathology practice, except visual inspection by light microscopy. In the current study, we evaluated the HE4 expression

in bladder tumors and although not significant statistically, the HE4 expression was proportionally higher in the invasive tumors than the noninvasive tumors (26% for the former and 12.5% for the latter).

There are studies conducted on endometrial carcinoma patients as to whether HE4 status is a predictor for muscle invasion in the literature. Kalogera and Prueksaritanond et al. have found that the serum HE4 level was correlated with deep myometrial invasion [14,15]. Minar et al. examined HE4 and its contribution to the preoperative surgical staging and found that serum HE4 level before operation predicted high-risk patients [16].

Only one study examined HE4 expression in bladder carcinoma in the literature (7). In this study, 9 out of 32 transitional cell carcinoma cases (28%) were stained with HE4. Their rate was close to our study and the staining intensity was weak in the majority of the cases, as seen in our study.

HE4 expression seen in our PUNLMP cases deserves attention. Higher expression rate seen in PUNLMP compared to overt malignant cases in this study can be explained with the low number of the study population in this group. On the other hand, HE4 positivity might have predicted PUNLMP cases that would progress to a higher grade lesion, as the long-term outcome of these cases demonstrates a broad range of recurrence and progression rates [17].

This study had some limitations, which had to be pointed out. The small patient population was the most important limitation. Secondly, cases from the urothelial proliferation of uncertain malignant potential were not included, as this category was introduced after the study period. Thirdly, the retrospective nature of the study did not allow us to measure the serum level of HE4 and to combine it with the study.

5. CONCLUSION

In conclusion, we observed that there was a trend towards statistical significance between invasive and non-invasive urothelial tumors. Further large-scale studies combining densitometric measurement of urine and serum level of HE4 are needed to determine whether it can be or cannot be used as a marker to assess invasion status when the bladder muscle is not sampled histopathologically.

CONSENT AND ETHICAL APPROVAL

As per university standard guideline participant consent and ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics. *CA Cancer J Clin.* 2017;67(1):7-30. DOI:10.3322/caac.21387.
2. Jebar AH, Hurst CD, Tomlinson DC, Johnston C, Taylor CF, Knowles MA. FGFR3 and Ras gene mutations are mutually exclusive genetic events in urothelial cell carcinoma. *Oncogene.* 2005;24(33):5218-25.
3. Lindgren D, Frigyesi A, Gudjonsson S, Sjö Dahl G, Hallden C, Chebil G, Veerla S, Ryden T, Månsson W, Liedberg F, Höglund M. Combined gene expression and genomic profiling define two intrinsic molecular subtypes of urothelial carcinoma and gene signatures for molecular grading and outcome. *Cancer Res.* 2010;70(9):3463-72.
4. Mitra AP. Molecular substratification of bladder cancer: Moving towards individualized patient management. *Ther Adv Urol.* 2016;8(3):215-33. DOI:10.1177/1756287216638981. Epub 2016 Mar 28.
5. Ma Q, Wang Q, Zhong D. Advances of human epididymis protein 4 in lung cancer. *Zhongguo Fei Ai Za Zhi.* 2015;18(3):184-6. DOI:10.3779/j.issn.1009-3419.2015.03.10
6. Bingle L, Cross SS, High AS, Wallace WA, Rassl D, Yuan G, et al. WFDC2 (HE4): a potential role in the innate immunity of the oral cavity and respiratory tract and the development of adenocarcinomas of the lung. *Respir Res.* 2006;7:61.
7. Galgano MT, Hampton GM, Frierson HF Jr. Comprehensive analysis of HE4 expression in normal and malignant human tissues. *Mod Pathol.* 2006;19(6):847-53
8. Mo D, He F. Serum Human Epididymis Secretory Protein 4 (HE4) is a Potential Prognostic Biomarker in Non-Small Cell Lung Cancer. *Clin Lab.* 2018;64(9):1421-1428. DOI:10.7754/Clin.Lab.2018.180222.
9. Luo CH, Zhao M, Tang YX, Shahabi S, Fang KN, Chen Y, Tang Y, Chen XY, Wang J, Zhou HH. Increased HE4 mRNA Expression Correlates with High Level Of eIF3a mRNA And Better Survival in

- Women with Epithelial Ovarian Cancer. J Cancer. 2018;9(6):1088-1095. DOI:10.7150/jca.23639. eCollection 2018.
10. Bulut T, Celik B, Yalcin AD, Keser S. Tissue expression of HE4 a and its correlation with CA125 and P53 in high grade serous ovarian carcinoma. Eur J Gynaecol Oncol. DOI:10.12892/ejgo3992.2017 (Epub ahead of print).
 11. Celik B, Bulut T, Yalcin AD. The expression of human epididymis protein 4a (HE4) in the normal gastric Epithelia and Its Role in the Development of Intestinal Metaplasia and Gastric Cancer. 2018; 27(2):1-10. DOI:10.9734/JAMMR/2018/41815
 12. Bulut T, Celik B. Tissue HE4 expression distinguishes pulmonary adenocarcinoma from squamous cell carcinoma and small cell carcinoma. AQCH. 2018;1:1-8.
 13. Humphrey PA, Moch H, Cubilla AL, Ulbright TM, Reuter VE. The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs-Part B: Prostate and Bladder Tumours. Eur Urol. 2016;70(1):106-119. DOI:10.1016/j.eururo.2016.02.028. Epub 2016 Mar 17.
 14. Kalogera E, Scholler N, Powless C, et al. Correlation of serum HE4 with tumor size and myometrial invasion in endometrial cancer. Gynecol Oncol. 2012;124(2):270-5. DOI:10.1016/j.ygyno.2011.10.025.
 15. Prueksaritanond N, Cheanpracha P, Yanaranop M. Association of serum HE4 with primary tumor diameter and depth of myometrial invasion in endometrial cancer patients at Rajavithi hospital. Asian Pac J Cancer Prev. 2016;17(3):1489-92.
 16. Minar L, Klabensova I, Jandakova E, Zlamal F, Bienertova-Vasku J. Prognostic value of human epididymis protein 4 in endometrial cancer and its utility for surgical staging. J Obstet Gynaecol Res. 2015;41(10):1644-52. DOI:10.1111/jog.12764.
 17. Maxwell JP, Wang C, Wiebe N, Yilmaz A, Trpkov K. Diagn Pathol. Long-term outcome of primary Papillary Urothelial Neoplasm of Low Malignant Potential (PUNLMP) including PUNLMP with inverted growth. 2015;10:3. DOI:10.1186/s13000-015-0234-z.

© 2018 Celik and Bulut; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
<http://www.sdiarticle3.com/review-history/45373>